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# The *Hansenula polymorpha PER8* Gene Encodes a Novel Peroxisomal Integral Membrane Protein Involved in Proliferation

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*Abstract.* We previously described the isolation of mutants of the methylotrophic yeast *Hansenula polymorpha* that are defective in peroxisome biogenesis. Here, we describe the characterization of one of these mutants, *per8*, and the cloning of the *PER8* gene. In either methanol or methylamine medium, conditions that normally induce the organelles, *per8* cells contain no peroxisome-like structures and peroxisomal enzymes are located in the cytosol. The sequence of *PER8* predicts that its product (Per8p) is a novel polypeptide of 34 kD, and antibodies against Per8p recognize a protein of 31 kD. Analysis of the primary sequence of Per8p revealed a 39-amino-acid cysteine-rich segment with similarity to the C3HC4 family of zinc-finger motifs. Overexpression of *PER8* results in a markedly enhanced increase in peroxisome numbers. We show that Per8p is an integral membrane protein of the peroxisome and that it is concentrated in the membranes of newly formed organelles. We propose that Per8p is a component of the molecular machinery that controls the proliferation of this organelle.



EUKARYOTIC cells are divided into a variety of membrane-bound compartments or organelles, each responsible for performing specific metabolic functions. To maintain each organelle, the cell must correctly direct specific sets of proteins to their proper subcellular locations and, as the cell grows and divides, the organelles must be duplicated. For some organelles, such as the endoplasmic reticulum, mitochondrion, and peroxisome, the cell can also increase organelle numbers in response to certain environmental stimuli (Bolender et al., 1973; Attardi et al., 1988; Braunbeck and Völkl, 1991; Dreyer et al., 1992; Luiken et al., 1992). Of these organelle biogenesis tasks, significant progress has been made in elucidating the molecular mechanisms responsible for protein sorting, whereas little is known regarding the mechanisms responsible for organelle duplication/proliferation.

We are interested in understanding biogenesis in peroxisomes (glyoxysomes, glycosomes), a class of single membrane-bound organelles that exists in virtually all cells and is the site of a number of important oxidative reactions (Borst, 1989; Veenhuis and Harder, 1991; van den Bosch, 1992; Subramani, 1993). An unusual characteristic of peroxisomes relative to other organelles is their functional diversity; that is, the specific metabolic pathways found in the organelle vary depending upon the organism, the tissue, and its environment. The importance of peroxisomes to humans is dramatically demonstrated by a family of lethal genetic diseases called Zellweger syndrome in which peroxisomes appear to be absent from or deficient in patient cells (Lazarow and Moser, 1989). Thus, peroxisomes are clearly essential for human survival.

In recent years, basic features of peroxisome biogenesis have emerged. Proteins destined for the peroxisome are synthesized on free ribosomes, usually at their mature sizes, and posttranslationally imported into the organelle (Roa and Blobel, 1983; Fujiki et al., 1986). For import of peroxisomal matrix enzymes, two distinct peroxisomal targeting signals (PTSs)[1] have been defined (for review see de Hoop and AB, 1992). The most common signal, PTS1, is a carboxy-terminal tripeptide that is typically SKL or a conservative variant (Gould et al., 1987, 1990; Aitchison et al., 1991; Keller et al., 1991). The second, PTS2, is found on only a few peroxisomal enzymes such as mammalian and yeast 3-ketoacyl-CoA thiolases and yeast amine oxidase (Osumi et al., 1991; Swinkels et al., 1991; Glover et al., 1994; Faber et al., 1995). PTS2 is located near the amino terminus and

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1. *Abbreviations used in this paper*: AMO, amine oxidase; AOX, alcohol oxidase; CAT, catalase; DHAS, dihydroxyacetone synthase; EM, electron microscopic; MAL, maltose binding protein; MOX, methanol oxidase; Mut$^+$, methanol-utilizing; Mut$^-$, methanol-utilization-defective; ORF, open reading frame; $P_{MOX}$, alcohol oxidase promoter; PTS, peroxisomal targeting signal.

is cleaved in some instances (rat thiolase) but not in others (yeast thiolase). Peroxisomal membrane proteins and some matrix enzymes do not appear to have either PTS; therefore, it is likely that one or more additional PTSs exist. Aside from targeting signals, import machinery components remain largely unidentified with the exception of the *Pichia pastoris* PAS8 gene product which may be a receptor for proteins bearing PTS1 (McCollum et al., 1993).

Less is known regarding the cell's ability to control peroxisome volume and number. Like mitochondria, peroxisomes are thought to duplicate by fission from preexisting organelles and to actively migrate into daughter cells (Veenhuis et al., 1979; Attardi and Schatz, 1988). The number of peroxisomes per cell can increase dramatically in response to metabolic needs. For example, certain hypolipidemic drugs such as clofibrate induce peroxisomes to proliferate in rat liver cells (Lock et al., 1989). However, the most extreme example of a proliferative response is observed in methylotrophic yeasts such as *Hansenula polymorpha* where methanol induces a massive increase in peroxisome size and number (Veenhuis et al., 1979). In glucose-grown cells of this yeast, only one or a few small peroxisomes are present, whereas in methanol, peroxisomes can account for as much as 80% of total cell volume. Interestingly, when peroxisomal enzymes are expressed at high levels in glucose-grown cells, the organelles increase in size but not number (Gödecke et al., 1989; Roggenkamp et al., 1989). Thus, it appears that peroxisome size may be a reflection of the amount of matrix protein, while proliferation is controlled by a separate mechanism.

*H. polymorpha* is an attractive model system for genetic studies on peroxisome biogenesis (Veenhuis et al., 1992). In this organism, detailed physiological, biochemical, and ultrastructural information exists on the role of peroxisomes in the metabolism of a variety of unusual carbon and nitrogen sources (Veenhuis and Harder, 1987, 1991). In addition, methods for classical- and molecular-genetic manipulation of the organism are well developed (Cregg, 1987; Gleeson and Sudbery, 1988; Faber et al., 1992; Titorenko et al., 1993). In previous reports, we have described the isolation of *H. polymorpha* mutants that are defective in peroxisome biogenesis (*per* mutants) (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In this report, we describe the characterization of one mutant, *per8*, and the molecular cloning and characterization of the *PER8* gene and its product. We show that *PER8* encodes a 34-kD peroxisomal integral membrane protein that appears to play a role in peroxisome proliferation.

## Materials and Methods

### Strains, Media, and Microbial Techniques

*H. polymorpha* strains used in this study are listed in Table I. *H. polymorpha* cultures were grown at 37°C in a complex medium (YPD) composed of 1% yeast extract, 2% peptone, and 2% dextrose, or in one of the following minimal media: YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate) supplemented with either 0.4% dextrose or 0.5% methanol and 0.05% yeast extract; MAD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.25% methylamine, 0.25% dextrose). Nutritional supplements for growth of auxotrophic strains were added to 50 μg/ml as required. Sporulation (mating) medium was composed of 2% malt extract.

*Table I. H. polymorpha Strains Used in This Study*

| Strain | Genotype | Source or reference |
|---|---|---|
| CBS4732 | wild type | CBS4732 |
| A16 | *leu1* | Veale et al. (1992) |
| C76 | *per8-1* | This study |
| CT100 | *per8-1 leu1* | This study |
| CT101 | *per8Δ::SLEU2* | This study |
| CT102 | *per8Δ::SLEU2 ade11* | This study |
| CT103 | *per8-1 leu1* $P_{MOX}$-*PER8* | This study |
| CT104 | *per1 leu1* | This study |

Genetic manipulations of *H. polymorpha* have been described (Gleeson and Sudbery, 1988; Cregg et al., 1990; Faber et al., 1992). Yeast transformations were performed by the Klebe procedure (Klebe et al., 1983). Cultivation of *Escherichia coli* strains and recombinant DNA techniques were performed as described (Sambrook et al., 1989).

### Cell Fractionation

Methanol-utilizing ($Mut^+$) strains were precultured in YPD medium to an $OD_{600}$ of ~0.75 and then shifted by centrifugation into one liter of YNB methanol medium at a starting $OD_{600}$ of 0.005. Methanol-utilization-defective ($Mut^-$) strains were precultured in one liter of YPD medium and the entire culture was shifted at an $OD_{600}$ of ~0.75 into one liter of methanol medium. Cultures were incubated for 16 h and harvested by centrifugation. For growth on methylamine, cultures were precultured in YNB dextrose and shifted by centrifugation into MAD medium at a starting density of 0.1 $OD_{600}$ units and harvested at an $OD_{600}$ of ~1.0. Cells were washed three times with 10 ml of distilled water and resuspended in 4 ml of digestion buffer (5 mM K 3-[N-morpholino] propanesulfonate [pH 7.2], 0.5 M KCl, 10 mM $Na_2SO_3$) per gram of cells. Cells were then converted to protoplasts by the addition of Zymolyase 100T (ICN Biomedicals, Costa Mesa, CA) to 0.25 mg/ml and incubated at 37°C for 30 to 40 min with occasional shaking. All subsequent steps were carried out at 4°C. Protoplasts were diluted with 10 vol of ice-cold 1 M sorbitol and centrifuged at 4,000 *g* for 10 min. The resulting pellets were gently resuspended in sorbitol-MES buffer (1 M sorbitol, 5 mM K 2-[N-morpholino]ethanesulfonic acid, pH 5.5) at 1 ml/gram of cells. An equal volume of 0.25 M sorbitol, 5 mM MES (pH 5.5) was then added to the suspension while gently shaking and the mixtures were placed on ice for 10 min. A volume of 1.75 M sorbitol, 5 mM MES (pH 5.5) was then added sufficient to bring the solution back to 1 M sorbitol and samples were centrifuged at 1,000 *g* for 20 min to remove unbroken cells and other debris. The supernatants were subjected to centrifugation at 25,000 *g* for 25 min and the resulting pellets were resuspended in 100 μl of sorbitol-MES buffer and, along with supernatant fractions, were examined for enzyme activities. For purification of peroxisomes, pellets resulting from the differential centrifugation procedure described above were layered over a discontinuous sucrose gradient, centrifuged, and fractions collected as described previously (Douma et al., 1985).

Extraction of peroxisomes with triethanolamine and carbonate were performed as described in Waterham et al. (1994). Triton X-114 extractions were done as previously described (Bordier, 1981). Urea extractions were performed as described (Cunningham et al., 1989) except that the 6 M urea solution was in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl.

### Genomic DNA Library Construction

The *H. polymorpha* genomic DNA library used to clone *PER8* was constructed by inserting total DNA from wild-type *H. polymorpha* strain CBS4732 into the *H. polymorpha–E. coli* shuttle plasmid pYT3. This plasmid was constructed by inserting the *Saccharomyces cerevisiae LEU2* (*SLEU2*) gene on a 2.2-kb SalI-XhoI fragment into the SalI site of pUC19. *SLEU2* serves as a selectable marker for transformation into *leu1* strains of *H. polymorpha* (Gleeson et al., 1986). pYT3 also contains an *H. polymorpha* NruI-PvuII DNA fragment of 2.5 kb that contains autonomous replication sequence (HARS) activity in *H. polymorpha* inserted into the SmaI site of pUC19. To construct the library, total DNA from *H. polymorpha* was extracted and partially digested with Sau3AI. Fragments of 5 to 10 kb were pooled and further size selected by sucrose density gradient centrifugation. To receive the DNA fragments, pYT3 was digested with BamHI and treated with calf intestine alkaline phosphatase. Approximately equal molar

amounts of fragmented DNA and vector were mixed and ligated with T4 DNA ligase. Ligation products were transformed into *E. coli* strain DH5α and approximately 29,000 colonies were collected. Plasmid DNA was extracted from these cells and purified as described (Sambrook et al., 1989). The library was stored as plasmid DNA in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA at −20°C.

## Isolation and Characterization of the PER8 Gene

Cells of *H. polymorpha* strain CT100 (*per8-1 leu1*) were transformed with the *H. polymorpha* DNA library and Leu$^+$ colonies were selected on YNB glucose medium agar plates. Approximately 2,000 Leu$^+$ transformants were washed from the plates and inoculated into liquid YNB methanol medium. Samples of the liquid culture were periodically spread onto YNB methanol medium agar plates. After 3 d, growth on methanol was observed and the culture was harvested. Total DNA was extracted from these cells and plasmids were recovered by transforming the DNA into *E. coli*. Plasmid DNA was isolated from 10 *E. coli* transformants and all were found to contain pYT3 with an insert of 5.8 kb. One of these plasmids, pYT7 (shown in Fig. 4) was retransformed into CT100 by selection for Leu$^+$. The resulting Leu$^+$ transformants were then replica plated on methanol medium agar plates and all were observed to also be Mut$^+$. To further define the location of *PER8* sequences, selected subfragments from pYT7 were subcloned into pYT3 and tested for Mut$^+$ complementation of CT100. An EcoRI-NruI fragment of 1.6 kb was identified and inserted into pBluescript II SK$^+$ (Stratagene Corp., La Jolla, CA) in both orientations and a series of subclones containing deletions of the fragment were generated by limited digestion with exonuclease III (Henikoff, 1987). The DNA sequence of both strands of the insert was then determined by the dideoxy chain-termination method (Sanger and Coulson, 1975). DNA and predicted protein sequences were analyzed using MacVector software (IBI, New Haven, CT) and compared with the GenBank data base.

## Construction of PER8 Disruption Strain

A vector designed to delete all but the first 68 amino acids of the predicted PER8 product (Per8p) was constructed in five steps. In the first, pBluescript II SK$^+$ was digested with EcoRI and KpnI and an adaptor oligonucleotide (AATTAGTCGACGAATTCAGTACTGGTAC and CAGTACTGAATTCGTCGACT) was inserted. This step resulted in the removal of pBluescript restriction sites between EcoRI and KpnI, destruction of the pBluescript EcoRI site, and generation of the following new restriction sites: SalI, EcoRI, ScaI, KpnI. The resulting vector was named pDT2. In the second step, a 520-bp fragment composed of sequences flanking the 5′ end of *PER8* plus sequences encoding the first 68 amino acids of the gene was amplified by the PCR method from plasmid pYT7 using the 5′ primer TTGGTACCAGTACTAGCCGCTAAAAAAACCGGGCGT and 3′ primer GGGTCGACGAATTCGGAACCCACCAATGTTGTGAGA. The PCR product was digested with EcoRI and KpnI and inserted into EcoRI-KpnI-digested pDT2 to make pDT3. In the third step, a second fragment of 630-bp composed of sequences starting immediately 3′ of the TAA translation stop codon of *PER8* was PCR amplified using the 5′ primer AAAGGGAACAAAAGCTGGAGCT and 3′ primer ATGGATCCTCTAGATTGTAAAACGACGGCCAGTGA. This 3′ PCR product was digested with XbaI and inserted into XbaI-digested pDT3 to make pDT4. For the fourth step, additional 3′ sequences from the *PER8* locus were added by digesting pDT4 with NruI and inserting a 1.7-kb NruI-EcoRV fragment to create pDT5. In the fifth step, a 2.2-kb SalI-XhoI fragment encoding the *S. cerevisiae LEU2* gene (*SLEU2*) was inserted into SalI-digested pDT5 between the two *PER8*-flanking fragments to make plasmid pDT6. This plasmid was digested with ScaI and NruI to generate a 3.2-kb fragment containing the *PER8*-deletion allele (*per8Δ*) and terminating in sequences flanking *PER8* as shown in Fig. 7 *A*. The *per8Δ* fragment was transformed into *H. polymorpha* strain A16 (*leu1*) by selection for Leu$^+$ and transformants were screened for ones which were Mut$^-$. DNAs from Leu$^+$ and Mut$^-$ transformants were then examined by the Southern hybridization method for proper targeting of the *per8Δ* fragment into the *PER8* locus as described in the results section and shown in Fig. 7 *B*.

## Preparation of Antibodies against the PER8 Product

Per8p was expressed in *E. coli* as a fusion with the maltose binding protein (MAL) using a kit supplied by New England Biolabs (Beverly, MA). To construct the strain, the MAL expression vector, pMAL-c2, was first modified to accept a *PER8*-containing DNA fragment in the proper reading frame by digesting the vector with EcoRI and HindIII, and inserting adaptor oligonucleotides (AATTCAAGCTTGGATCCCCTTGG and AGCTGCAAGGGGATCCAAGCTTG) to make pFT2. Insertion of this adaptor resulted in the following changes in order: (*a*) restoration of the EcoRI site of pMAL-c2; (*b*) addition of a new HindIII site that was in frame with the HindIII site located near the 5′ end of *PER8* coding sequences; (*c*) addition of new BamHI and StyI sites; and (*d*) destruction of the original HindIII site of pMAL-c2. A 1.1-kb HindIII-StyI fragment encoding Per8p amino acids 3 through 295 was isolated from pYT7 and inserted into HindIII- and StyI-digested pFT2. Proper in-frame fusion of *malE-PER8* sequences was confirmed by DNA sequencing one of the resulting plasmids, pFT4. This plasmid was then transformed into *E. coli* strain TB1. A transformant was selected and grown and MAL-Per8p fusion protein was induced by addition of 0.3 mM isopropylthiogalactoside. After 2 h at 37°C, cells were harvested, lysed by sonication, and the fusion protein was purified by amylose affinity and DEAE-Sepharose chromatography as recommended by the supplier.

Purified MAL-Per8p fusion protein was used to immunize rabbits (Josman Laboratories, San Jose, CA). The resulting antiserum was affinity purified by a described procedure (Raymond et al., 1990). Crude serum was loaded onto a MAL-Per8p fusion protein column and bound antibodies were eluted with 0.2 M glycine (pH 2.4). The resulting preparation was then passed through a column containing total *H. polymorpha* protein from the *per8Δ* strain CT101 and another column containing MAL. The resulting antibody preparation was concentrated using a Centriprep-10 concentrator (Amicon Corp., Beverly, MA) and stored at −70°C in 1× PBS buffer (Sambrook et al., 1989).

## Construction of PER8 Overexpression Strain

Strain CT103, which overexpresses *PER8* in methanol medium, was constructed by transformation of *per8-1 leu1* strain CT100 with plasmid pET4. This plasmid was created from YIp32, a plasmid composed of a 2.2-kb SalI-XhoI fragment encoding the *S. cerevisiae LEU2* gene inserted at the SalI site of pBR322. To construct pET4, the HindIII site of YIp32 was first destroyed by digesting the plasmid with HindIII, filling in the resulting termini with dNTPs using Klenow fragment of DNA polymerase I and ligating to create YIp32-HK. Second, a 2.6-kb BamHI-BglII fragment that contains the *H. polymorpha* alcohol oxidase promoter ($P_{MOX}$) was inserted from plasmid pHIPX2 (Faber et al., 1992). This fragment was inserted into the BamHI site of YIP32-HK to make pET1. Third, pET1 was digested with HindIII and SmaI, and an adaptor oligonucleotide (AGCTGATGTTTAAGCTTTCGCGACCC and GGGTCGCGAAAGCTTAAACATC) was inserted to make pET3. The adaptor made the following changes in order: (*a*) destruction of the HindIII site at the junction of the adaptor and pET3; (*b*) addition of sequences encoding the methionine initiator ATG and the second amino acid of Per8p; (*c*) addition of a new HindIII in frame with the 5′ HindIII site of *PER8*; and (*d*) addition of an NruI site. Finally, a 1.3-kb HindIII-NruI fragment encoding all but the first two amino acids of the PER8 gene product was inserted into HindIII- and SmaI-digested pET3. The resulting plasmid pET4 contains the *PER8* gene under methanol oxidase (MOX) promoter control and was digested with StuI prior to transformation into CT100 to direct the plasmid to integrate at the $P_{MOX}$ locus. Proper integration of pET4 was confirmed by Southern blot analysis.

## Miscellaneous Methods

Total protein in samples was measured by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976). Alcohol oxidase (AOX) (van der Klei et al., 1990), catalase (Ueda et al., 1990), AMO (Zwart et al., 1980), and fumarase (Tolbert, 1974) activities were measured according to published procedures. Northern and Southern procedures were as described (Sambrook et al., 1989). Immunoblotting was performed using either the ProtoBlot system of Promega (Madison, WI) or the ECL system of Amersham Corp. (Arlington Heights, IL) as directed by the suppliers. Electron microscopy and immunogold-electron microscopy with anti-Per8p rabbit antibodies were performed as described (Waterham et al., 1992).

# Results

## per8 Cells Lack Morphologically Recognizable Peroxisomes

*H. polymorpha* strain C76 (*per8-1*) was one of a number of mutants isolated by screening a collection of Mut$^-$ strains

for ones in which intact peroxisomes were absent (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In methanol-grown wild-type *H. polymorpha*, peroxisomes are abundant (Fig. 1 *A*). In contrast, an exhaustive electron microscopic (EM) search of serially sectioned cells of the *per8-1* strain induced on methanol failed to reveal any peroxisome-like structures (Fig. 1 *B*). Thus, in *per8-1* cells, either peroxisomes were not present, or were morphologically unrecognizable, a phenotype we refer to as Per$^-$ (Titorenko et al., 1993). A striking feature of methanol-induced *per8-1* cells was the presence of a single large cytosolic crystalloid that was readily observed by phase contrast light microscopy as a bright yellow cubic structure tumbling within each cell (Fig. 1 *B, inset*). This crystalloid structure was primarily composed of AOX (Fig. 1 *C*) and is a common characteristic of most *H. polymorpha per* mutants examined to date (van der Klei et al., 1991).

Additional evidence for the absence of peroxisomes in *per8-1* was obtained from biochemical experiments designed to determine the location of peroxisomal enzymes. Enzyme activity measurements of extracts prepared from methanol-induced *per8-1* cells indicated that catalase (CAT) and AOX were present at substantial levels (Fig. 2 *A*). The third peroxisomal methanol-pathway enzyme, dihydroxyacetone synthase (DHAS), was also present as demonstrated by immunoblotting using antibodies against DHAS (data not shown). The location of these enzymes was investigated by subcellular fractionation of homogenized *per8-1* spheroplasts. After low-speed centrifugation to remove remaining whole cells, protoplasts, and nuclei, small organelles (primarily peroxisomes and mitochondria) were sedimented by centrifugation at 25,000 *g*. At this force, a major portion of the activity for peroxisomal enzymes in wild-type cells was present in the organelle pellet (Fig. 2 *B*). However, a significant amount of

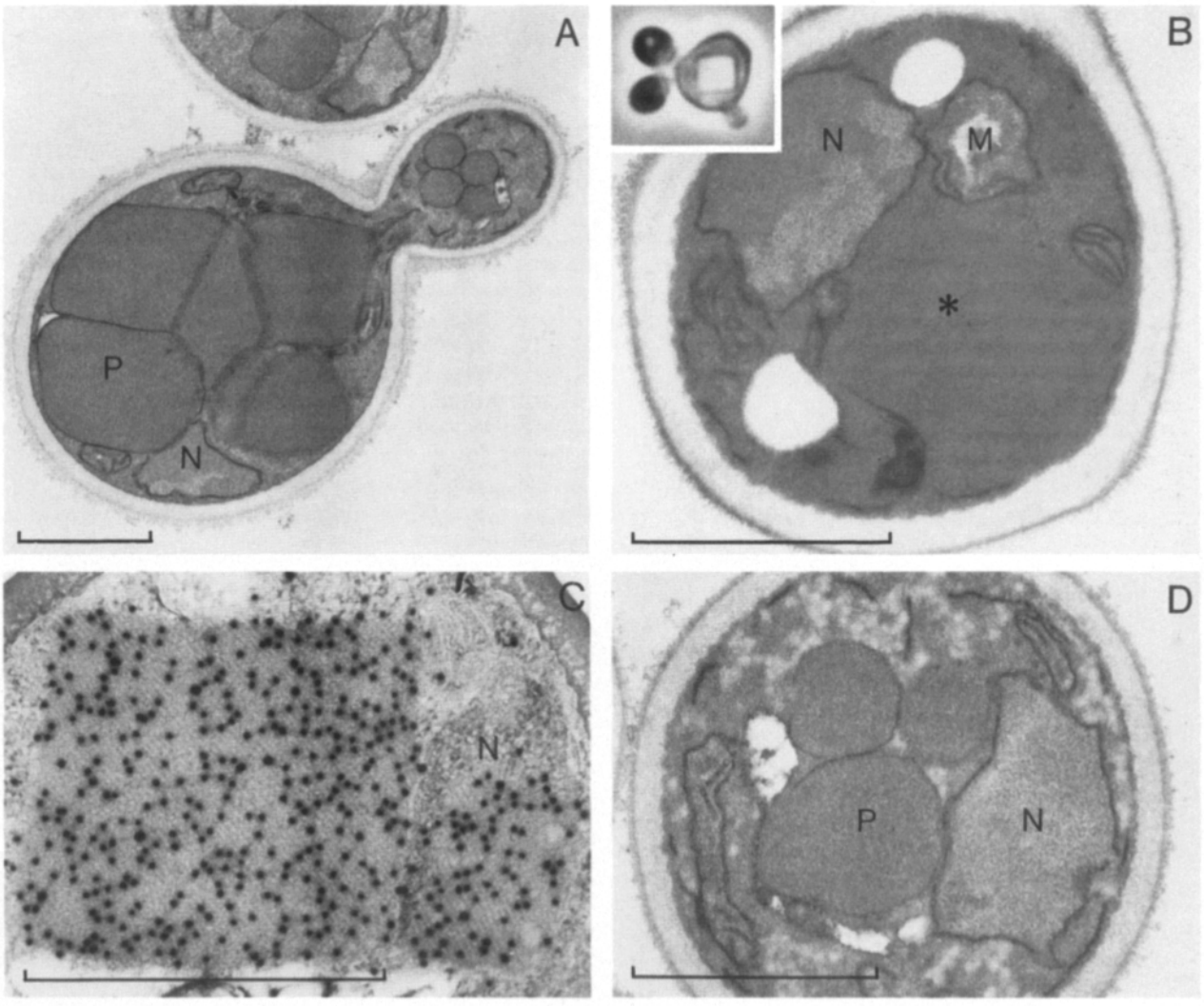



*Figure 1.* Electron micrographs showing subcellular morphology of selected *H. polymorpha* strains. (*A*) Methanol-grown wild-type cells contain numerous large peroxisomes (*P*); mitochondrion (*M*); nucleus (*N*). (*B*) Methanol-induced *per8-1* cells contain a large cytosolic AOX crystalloid (*) but no peroxisomes; (*inset*, *B*) phase-contrast light micrograph of *per8-1*. (*C*) Immuno-gold labeling of a methanol-induced *per8-1* cell with anti-AOX antibodies showing the presence of AOX in the cytosolic crystalloid. A second small crystalloid is also present in the nucleus of this cell, a common occurrence in *per8-1* and other *per* cells. (*D*) *per8-1* cells transformed with a complementing DNA fragment grow on methanol and contain normal peroxisomes. Bar, 1.0 μm.

activity was also present in the supernatant due to enzyme leakage or organelle breakage or both. In contrast, after centrifugation of *per8-1* preparations, almost all AOX and CAT activity and DHAS protein were located in the supernatant, indicating that these enzymes were located in the cytosol (Fig. 2 *B*). These results could not be due to unequal handling of *per8* cells during the fractionation procedures since fumarase, a mitochondrial marker enzyme, remained primarily in the pellet.

In *H. polymorpha*, the utilization of methylamine as a nitrogen source requires the peroxisomal enzyme amine oxidase (AMO) (Zwart et al., 1980; Sulter et al., 1990). AMO has recently been shown to be directed to peroxisomes via a PTS2 sequence (Faber et al., 1995), whereas AOX, CAT, and DHAS each contain a PTS1 sequence (Didion and Roggenkamp, 1992; Hansen et al., 1992). Thus, the fate of AMO is an indicator of PTS2 system function. In methylamine-grown *per8-1* cells, AMO and CAT activity levels were greater than twice those in wild-type cells (Fig. 2 *A*). After differential centrifugation, AMO activity was present primarily in the pellet fraction while CAT was mostly in the supernatant fraction (Fig 2 *B*). These results suggested that AMO may be in peroxisomes and therefore, that *per8-1* cells may be specifically defective in the import of PTS1-containing enzymes. On the other hand, AMO has been shown to form cytosolic aggregates in methylamine-grown cells of some *per* mutants (Sulter et al., 1990). Therefore, it was possible that in *per8-1* cells, AMO also formed aggregates and that these aggregates sedimented during differential centrifugation. To differentiate between these explanations, the pellet fractions resulting from differential centrifugation were subjected to sucrose density gradient centrifugation. As shown in Fig. 3 *A*, AMO from *per8-1* cells sedimented to a significantly higher density than peroxisome-enclosed AMO from wild-type cells, suggesting that, in *per8-1*, AMO was in a structure with little or no associated membrane such as a protein aggregate. Methylamine-grown *per8-1* cells were also examined by EM for peroxisomes. As with methanol-induced cells, no peroxisome-like structures could be found (data not shown). In addition, immunocytochemical results using antibodies against AMO showed that AMO was indeed located primarily in aggregates (Fig. 3 *B*). We concluded that AMO, like the other peroxisomal enzymes, was not in peroxisomes and that the absence of peroxisomes in *per8-1* cells was not a methanol- or PTS1-specific defect but a general impairment in peroxisome biogenesis.

A

| | *PER8* | *per8-1* | *per8-1+PER8* | *per8Δ* |
|---|---|---|---|---|
| Methanol | | | | |
| CAT | 100 | 121 | 73 | 90 |
| AOX | 100 | 34 | 57 | 22 |
| Methylamine | | | | |
| CAT | 100 | 368 | 120 | 399 |
| AMO | 100 | 253 | 108 | 233 |

B

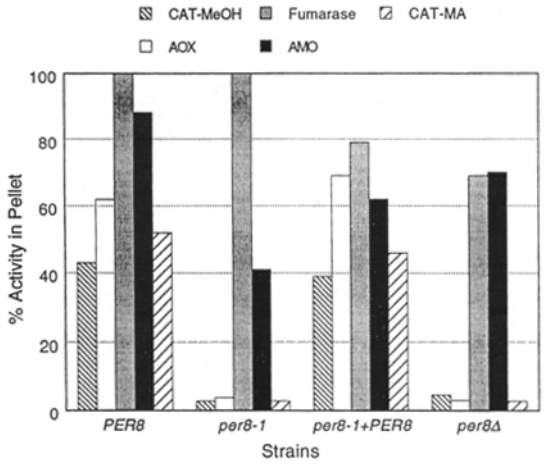


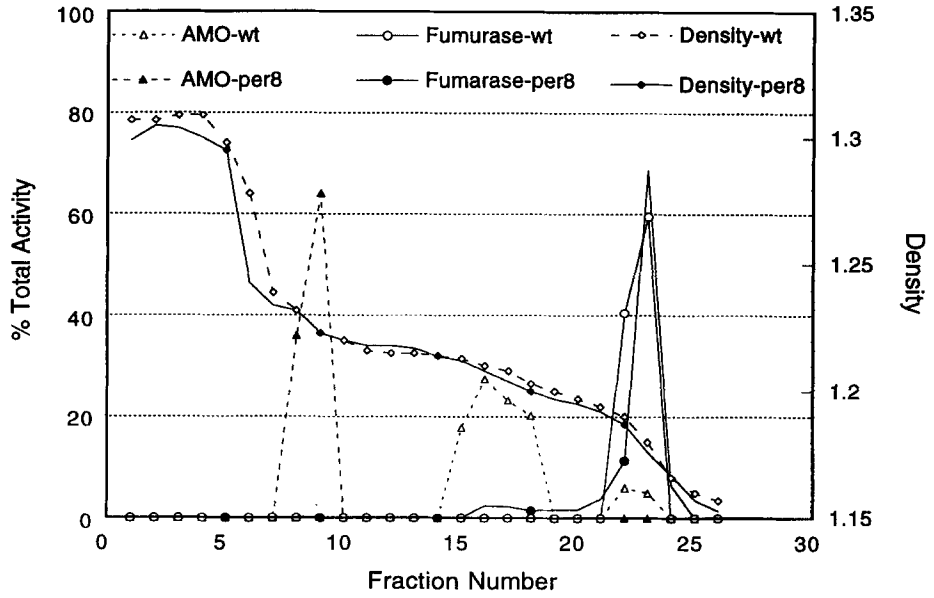

*Figure 2. per8* cells induce peroxisomal enzymes but do not import them into peroxisomes. (*A*) AOX and $CAT_{MeOH}$ activities in methanol-induced strains and AMO and $CAT_{MA}$ activities in methylamine-grown strains reported as a percentage of that in wild-type (*PER8*) cells. *per8-1* + *PER8* is strain CT100 (*per8-1 leu1*) transformed with *PER8*-containing plasmid pYT7. (*B*) Percentage of enzyme activity in post-25,000 *g* organelle pellet after differential centrifugation of homogenized protoplasts.

## *Isolation and Characterization of the PER8 Gene*

The *PER8* gene was isolated from an *H. polymorpha* genomic DNA library by functional complementation of *per8* strain CT100 (*per8-1 leu1*). Library transformants were initially selected for leucine prototrophy ($Leu^+$) on glucose medium and subsequently selected for growth on methanol ($Mut^+$). Total DNA was extracted from a pool of $Leu^+$ $Mut^+$ transformants and plasmids were recovered by transforming the DNA into *E. coli*. A plasmid, designated pYT7 (Fig. 4), was isolated that co-transformed the *per8-1 leu1* strain to both $Leu^+$ and $Mut^+$ simultaneously. *per8-1* cells transformed with pYT7 contained normal-appearing peroxisomes (Fig. 1 *D*) and sedimentable activity for AOX, CAT, and AMO (Fig. 2 *B*). Restriction mapping of pYT7 revealed an *H. polymorpha* DNA insert of 5.8 kb. By subcloning selected sub-fragments from pYT7, the complementing activity was located within a 1.6-kb EcoRI-NruI fragment. In northern blots, the 1.6-kb fragment hybridized to a single transcript of 1.4 kb that was present at low levels in glucose-grown wild-type *H. polymorpha* and induced approximately five-fold in methanol-grown cells (Fig. 5, lanes *4* and *5*). Since methanol induces peroxisome proliferation, the higher level of this transcript in methanol-grown cells was consistent with it being the product of a peroxisomal gene.

The DNA sequence of the 1.6-kb fragment from pYT7 revealed a single long open reading frame (ORF) of 885 bp with the potential of encoding a polypeptide of 295 amino acids (~34 kD) (Fig. 6 *A*). Several results indicated that this ORF encoded the PER8 gene product. First, as described in the next section, an *H. polymorpha* strain in which most of the ORF was deleted had the same phenotype as the *per8-1* strain and was an allele of *per8-1*. Second, antibodies prepared against the product of the ORF specifically reacted with a protein of 31 kD (see Fig. 11 *A*), a mass that was in approximate agreement with the mass predicted from the ORF. Translation of Per8p most likely begins at the assigned ATG since initiation at the next ATG in the ORF was predicted to generate a protein of only ~19 kD. However, since the apparent mass was less than the predicted mass,

A




B

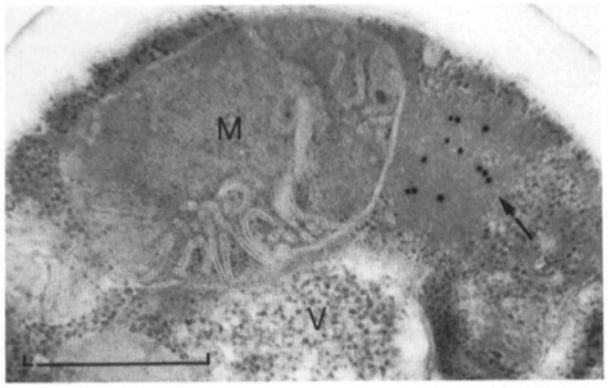



*Figure 3.* AMO is located primarily in protein aggregates in *per8-1* cells. (*A*) Sucrose density gradient profile of AMO activity in crude organelle pellet derived from methylamine-grown *per8-1* and wild-type cells. (*B*) Section of methylamine-grown *per8-1* cell after immuno-gold labeling with anti-AMO antibodies. Bar, 0.5 μm.

it was conceivable that Per8p may be posttranslationally processed. Third, as described below, the product of the ORF is a peroxisomal protein. Hydropathy analysis indicated that Per8p was of average overall hydrophobicity with no apparent α-helical transmembrane domains (Fig. 6 *B*). Database searches revealed no other proteins with overall sequence similarity to Per8p. The only identifiable feature of the predicted Per8p sequence was a segment of 39 amino acids near its carboxy-terminus that was a perfect match to the C3HC4 motif ($\underline{C}X_2\underline{C}X_{11\text{-}30}\underline{C}X\underline{H}X_2\underline{C}X_2\underline{C}X_{10\text{-}18}\underline{C}PX\underline{C}$), a subgroup within the cysteine-rich zinc-finger domain family (Fig. 6, *A* and *C*) (Freemont et al., 1991). Outside the C3HC4 region, the Per8p sequence displayed no further similarity to any of these proteins.

### *A PER8-deleted Strain Is Peroxisome Deficient*

An *H. polymorpha* strain in which most of *PER8* was deleted was created by the gene replacement method (Rothstein, 1983). For the replacement, a plasmid was constructed in which 700 bp of *PER8* coding sequence (nucleotides 206 to 906 encoding amino acids 69 to 295 in Fig. 6 *A*) was removed and replaced with a fragment containing the *S. cerevisiae LEU2* gene. This plasmid was then digested with a restriction enzyme to release the *PER8*-deletion allele (*per8*Δ) on the linear DNA fragment shown in Fig. 7 *A*, and introduced into *H. polymorpha* strain A16 (*leu1*). Transformants in which the fragment had deleted the *PER8* locus were isolated by selecting for Leu$^+$ colonies and then screening for ones which were also Mut$^-$. Proper targeting of the fragment was confirmed by Southern blot analysis (Fig. 7 *B*). For example, P2, a labeled probe composed of sequences located entirely within the deleted region, hybridized to a 8.5-kb fragment from wild-type *H. polymorpha* but did not hybridize with DNAs from *per8*Δ strains (Fig. 7 *B*, P2 lanes *2* and *3*). Furthermore, northern blots showed that methanol-induced cells of *per8*Δ strains no longer produced the 1.4-kb *PER8* message (Fig. 5 *B*, lane *2*). One *per8*Δ-derived

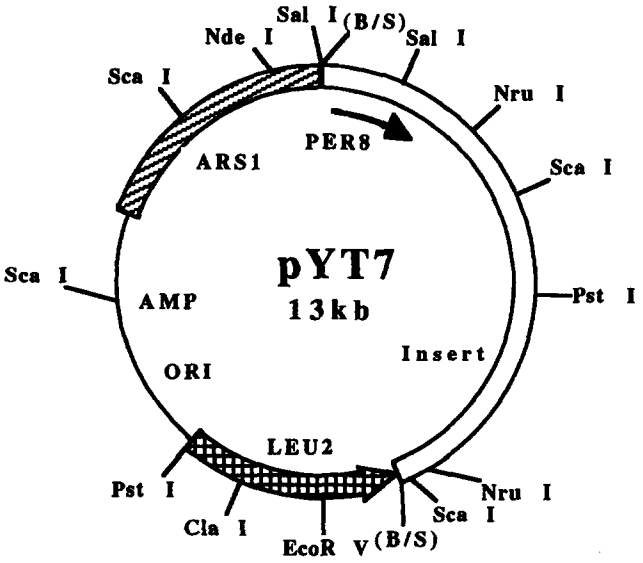



*Figure 4.* Restriction endonuclease cleavage site map of *PER8*-containing plasmid pYT7.

strain, CT102 (*per8Δ::SLEU2 leu1 ade11*), was crossed with CT100 (*per8-1 leu1*) by selection for growth on minimal glucose plates. The resulting diploids were then tested for methanol growth and were Mut⁻. In contrast, diploids from a control cross of CT100 and CT104 (*per1 leu1*) were Mut⁺. The *per8-1/per8Δ* diploid strains were sporulated and several thousand of their progeny were grown on glucose medium and tested by the replica plate technique for methanol growth. All spore progeny were Mut⁻, indicating that *per8-1* and *per8Δ* were tightly linked. The failure of *per8-1/per8Δ* diploids or their progeny to grow on methanol demonstrated that *per8-1* and *per8Δ* were likely to be mutant alleles of the same gene and therefore, that the cloned DNA fragment under investigation contained the same gene that was defective in *per8-1* and not a suppressor gene.

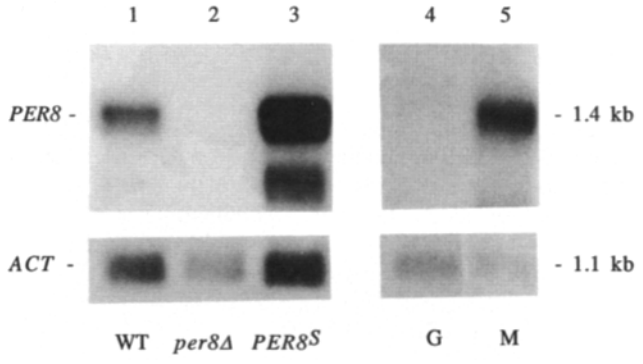
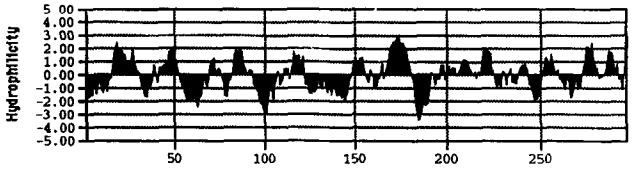



*Figure 5.* Northern blot showing induction of *PER8* message levels by methanol. All lanes contain 6 μg of total RNA. Methanol-grown wild-type cells contain a 1.4-kb *PER8* message (lane *1*) that is not present in methanol-induced *per8Δ* cells (lane 2) but is present at a high level in methanol-grown cells of a strain that expresses *PER8* under control of $P_{MOX}$ (lane *3*). (lanes *4* and *5*) *PER8* message level in glucose- and methanol-grown wild-type *H. polymorpha* cells, respectively. As a control, filters were hybridized with a labeled DNA fragment encoding the *S. cerevisiae* actin gene (*ACT*). *ACT* message levels in methanol-grown cells are approximately threefold lower than in glucose-grown cells.

A

```
-324                                                                    atcatcat
-316  caagccgctaaaaaaaccgggcgtgccttcgtctccggtagtggaagagactcccagccgagcagaaacgcccacgggg
-237  agcaaggcgcgggcaagtggtctttcgggtgagcttgatcgctcaggaagaccgtgcaagcggtggaccaaaaagacac
-158  gtgtgttcaagactttcagcggctgggacgtcgaggttgggatctgggagaccgtgtacgtagggttttgcttttgttct
 -79  atgtacgatttcactaaaaatatcaattacgaatgggctaatatttgcgtctggctaggctaaataattattcctttttt

   1  ATG TTT AAG CTT TTG TCT TTT GCC AAT GCT CCA GCG ATT GTG CGG GCC AAC CAG AAG GAT
   1   M  F  K  L  L  S  F  A  N  A  P  A  I  V  R  A  N  Q  K  D

  61  TCA TAT TTT GAG TCG AGG CTG CAC AAC CAG CTC TTA GAC GTT GTG AAG GCC ATC AAA GGC
  21   S  Y  F  E  S  R  L  H  N  Q  L  L  D  V  V  K  A  I  K  G

 121  TCT CAC TTT GTC CAC AAG TAC CCG GAG GAG CTG CGA ACC CTG GCC ACG GCT CTT TAT CTG
  41   S  H  F  V  H  K  Y  P  E  E  L  R  T  L  A  T  A  L  Y  L

 181  TGT CTC ACA ACA TTG GTG GGT TCC AAG ACG CTG GGC GAG GAG TAC GTG GAT CTG GTG TAT
  61   C  L  T  T  L  V  G  S  K  T  L  G  E  E  Y  V  D  L  V  Y

 241  GTG AGT CGT GAC GGA CGC AAG ATA CCG AAA TTT GCG TCC CGA TTT GGC TTT GTC GTG GCG
  81   V  S  R  D  G  R  K  I  P  K  F  A  S  R  F  G  F  V  V  A

 301  TAT GTG CTC TTC CCC TAT GCT GTT CGC CAA CTG CTT CAA AAA CTG AAA GCA CAG CAA AGC
 101   Y  V  L  F  P  Y  A  V  R  Q  L  L  Q  K  L  K  A  Q  Q  S

 361  CGG CTA GCA CAA CTG GTT TCC GGA GTC AGC TAC ATG AAC GTG ATG GAT CTT TTG AAT CTC
 121   R  L  A  Q  L  V  S  G  V  S  Y  M  N  V  M  D  L  L  N  L

 421  CAT CTG GCG CTC TTC TAC TTC ACG GGC AAA TAC TAT CAG TTT GCG AAA AGA CTC TTT GGT
 141   H  L  A  L  F  Y  F  T  G  K  Y  Y  Q  F  A  K  R  L  F  G

 481  CTG CGA TAT GCG TTT GGC TAT CGA GTC GAC AAA AAT CAG CAG AGA GCG CGA GGC AAC TAC
 161   L  R  Y  A  F  G  Y  R  V  D  K  N  Q  Q  R  A  R  G  N  Y

 541  GAG TTG CTG GGA CTG CTG ATC ATT TTT CAG ACA GTC TTC AAG AAC GTG GCA AAC TTG CGA
 181   E  L  L  G  L  L  I  I  F  Q  T  V  F  K  N  V  A  N  L  R

 601  AAA CTG TGG GGG GCC ACC AAG ACC GTG CAG GAC TCG GGA GAT TTA ATC TAC AGG TTC AGA
 201   K  L  W  G  A  T  K  T  V  Q  D  S  G  D  L  I  Y  R  F  R

 661  GAC CAG ACG AGC GAC GTT ATA GAC CTT GCA GAC CCA AAG GTG CTG CCG TAC CTG CCT GAG
 221   D  Q  T  S  D  V  I  D  L  A  D  P  K  V  L  P  Y  L  P  E

 721  GCT TCG CGC ACG TGC ATG CTG TGT CTG TCG CCG ATG AAA GAC CCA TCA TGC GGC GAG TGC
 241   A  S  R  T  C  M  L  C  L  S  P  M  K  D  P  S  C  G  E  C

 781  GGC CAC GTC TTC TGC TGG AAG TGC GTG CTG GAC TGG GTC AAG GAG CGC CAG GAG TGT CCG
 261   G  H  V  F  C  W  K  C  V  L  D  W  V  K  E  R  Q  E  C  P

 841  TTG TGC AGG GCC AAA ATG AGA GAG TCG CAG CTG TTG CCT CTA CGA TAA gtgtctatgaacaat
 281   L  C  R  A  K  M  R  E  S  Q  L  L  P  L  R  ***

 904  acataagggccacggatcagtttctattaacacccattcggaacagcacctgcttgcccttcttgcccttttctgatctc
 983  gaagtcgggcttcttcgcgtcgttgctgtaagccctgacccccattgtccgatcgaggtgttgaccgggttgacccttggt
1062  ggcttgggtttctgggcactgggcagtttcgggaacagctggtcgtccagagccgggggcttgtggcttgggctgctaat
1141  tgttgaggtacgtcagccgcacgtttggcgaggtgcctctattgacgacaggcttcaacttcgggggcgcgctttgcgc
1220  agccccgttgtgggctggttctggccccacggtcccacgggccgcccgtggcgcagtgctgagcgcagggaactgctcg
1299  tccagcagcttttcg
```

B




C

```
Per8p          SRTCMLC LSPMKDPSCGE   CGHVFCWKCVLD   WVKERQ   ECPLCRAK
Pas4p          SRKCILC LMNMSDPSCAP   CGHLFCWSCLMS   WCKERP   ECPLCRQH
PAF-1p         GKECALCGEWPTMPHTIG    CEHIFCYFCAKS   SFLFDVY  FTCPKCGTE
Pep5p          NQTCPMCRLTLDIPVVFFK   CGHIYHQHCLNEEEDTLESERKLFKCPKCLVD
PS 43K         ELYCGLCGESIGERNSRLQALPCSHIFHLRCLQ    NNGTRS    CPNCRRS
consensus        CXIC    11-30X      CXHXIPCXXCL    10-18X     CPXC
                  V                    YH  I
                  L                    L
```

*Figure 6.* (*A*) Nucleotide and predicted amino acid sequences of *PER8*. The cysteine-rich motif is located from residue 245 through 282 in Per8p and is underlined. (*B*) Hydrophilicity plot of Per8p-predicted primary structure shows that the peptide is of average hydrophobicity and lacks potential α-helical membrane-spanning domains. (*C*) Alignment of C3HC4 motifs in selected proteins. Postsynaptic 43K protein (*PS 43K*). These sequence data are available from EMBL/GenBank/DDJB under accession number Z38001.

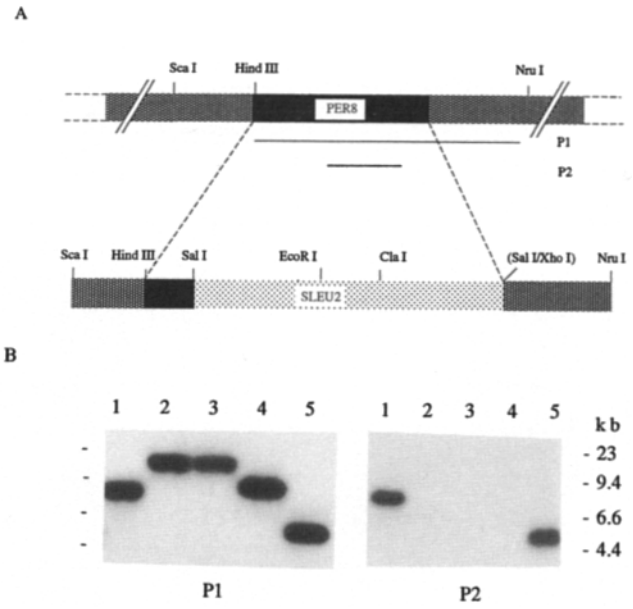



*Figure 7.* Deletion of the *PER8* gene. (*A*) *PER8* DNA sequences encoding Per8p amino acids 69 through 295 were replaced with a DNA fragment containing the *S. cerevisiae LEU2* gene and inserted into the *H. polymorpha* genome by homologous recombination. (*B*) Correct targeting of the *per8*Δ fragment was demonstrated by Southern blotting of genomic DNAs cut with NruI and hybridizing with labeled fragments P1 and P2 indicated in *A*. (lanes *1*) 2 μg of wild-type genomic DNA; (lanes *2* and *3*) 2 μg of DNA from each of two *per8*Δ strains; (lanes *4*) 2 ng of linear DNA from *per8*Δ plasmid pDT6; (lanes *5*) 2 ng of linear DNA from *PER8* plasmid pBS1.

Phenotypically, the *per8*Δ strain was indistinguishable from *per8-1*. In addition to being Mut⁻, EM examination of methanol-induced *per8*Δ cells showed that they were devoid of peroxisomes and contained the cytosolic AOX crystalloid that is typical of *H. polymorpha per* mutants (not shown). In addition, peroxisomal enzymes in *per8*Δ cells were induced to normal levels but were mislocalized to the cytosol as judged by subcellular fractionation results (Fig. 2).

### *Per8p Overexpression Leads to Enhanced Peroxisome Proliferation*

To gain insight into Per8p function, the effect of overexpression of *PER8* on the cell's morphology was examined. For these experiments, an *H. polymorpha* strain was constructed that expressed *PER8* under the transcriptional control of the methanol-regulated and highly efficient $P_{MOX}$. In methanol-grown cells of this strain (CT103; *leu1 per8-1* $P_{MOX}$-*PER8*), the level of *PER8* message was approximately 10 times higher than in wild-type cells (Fig. 5, lanes *1* and *3*). As described in the following section, immunoblots showed a similar increase in the level of Per8p as well. Expression from $P_{MOX}$ is highly repressed by glucose; therefore, as expected, most glucose-grown CT103 cells contained only a single small peroxisome (Figs. 8 *A* and 9), similar to that in wild-type cells.

Detailed EM examination of $P_{MOX}$-*PER8* cells harvested at selected times after methanol induction revealed an interesting temporal series of events. Within the first few hours after shift, the single peroxisome enlarged severalfold (Figs. 8 *B* and 9), a phenomenon also observed in wild-type cells

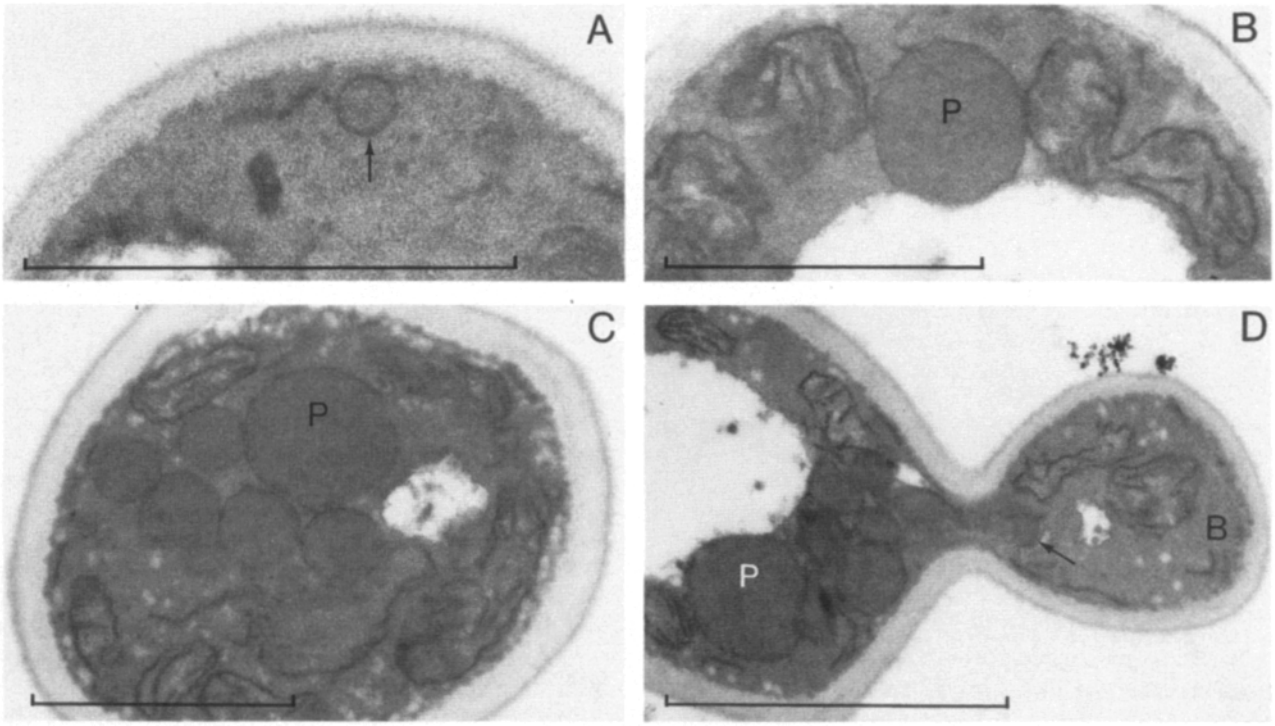



*Figure 8.* Electron micrographs showing enhanced peroxisome proliferation in $P_{MOX}$-*PER8* strain. (*A*) Single small peroxisome in glucose-grown $P_{MOX}$-*PER8* cell. (*B*) 3 h after shift to methanol medium peroxisome has grown but not proliferated. (*C*) Enhanced proliferation in cells after 6 h in methanol. (*D*) Segregation of peroxisomes to bud at 6 h. Bar, 1.0 μm.

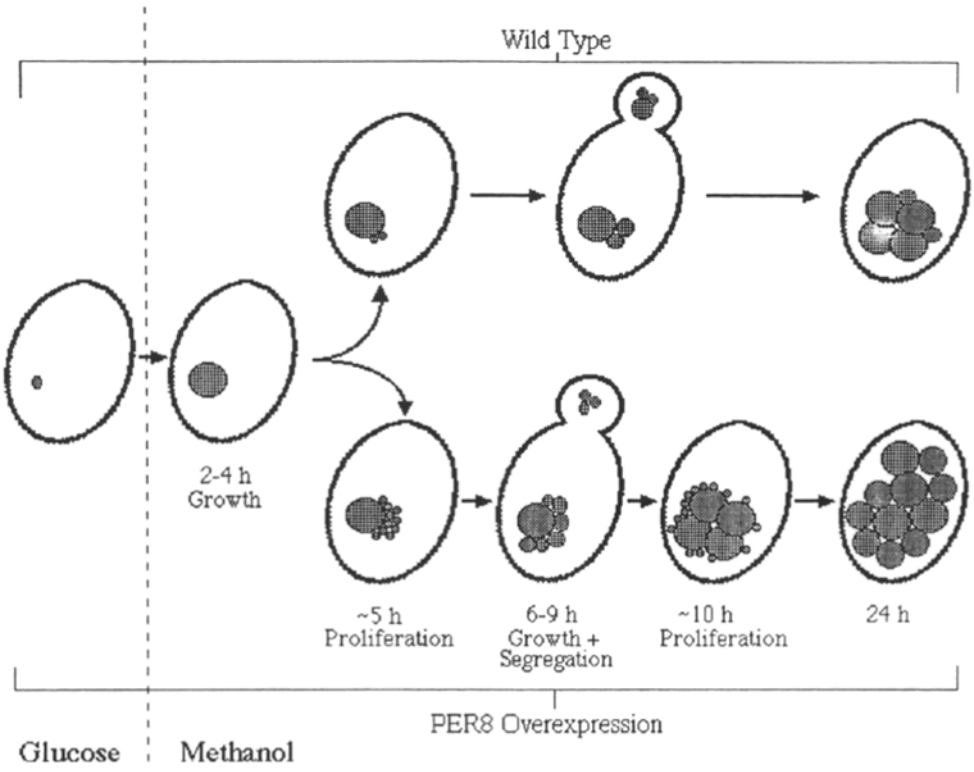



*Figure 9.* Diagram of peroxisome proliferation events in $P_{MOX}$-*PER8* overexpression strain and wild-type *H. polymorpha* after shift to methanol medium.

and due to the import of newly synthesized AOX, CAT, and DHAS (Veenhuis and Harder, 1991). Between 4 to 6 h postshift, an enhanced rate of peroxisome proliferation was observed, resulting in the development of 5 to 10 new organelles (Figs. 8 *C* and 9), where, in wild-type cells, on average two new ones were formed. The newly formed peroxisomes consistently appeared at a single location on the parent organelle and often in close association with endoplasmic reticulum (Fig. 10 *A*). Over the next several hours, the small newly formed organelles increased in size and some appeared to migrate into newly formed cell buds (Figs. 8 *D* and 9). However, the number of peroxisomes migrating into buds of the Per8p overexpression cells was not significantly greater than that observed in wild-type cell buds. Between 8 and 10 h postshift, a second proliferation round was observed that appeared to originate from one or two of the organelles that had been generated during the first round. This led to a further increase in peroxisome numbers (Figs. 9 and 10 *C*). Thus, the increased proliferation of peroxisomes in response to Per8p overexpression indicated that Per8p plays an important role in regulating the number of peroxisomes generated during proliferation. That proliferation did not occur continuously suggested that other factors determine the timing of these events.

### *Per8p Is a Peroxisomal Integral Membrane Protein*

Per8p was characterized through rabbit polyclonal antibodies raised against Per8p expressed in *E. coli* as a fusion with maltose-binding protein. The crude anti-Per8p antibody serum recognized a 31-kD polypeptide in immunoblots prepared from methanol-grown wild-type and *per8-1* extracts that was not present in extracts from methanol-induced *per8*Δ cells (Fig. 11 *A*, lanes *1*, *2*, and *4*). The apparent molecular weight of Per8p was in reasonable agreement with the 34-kD weight calculated from the predicted primary sequence. The putative Per8p band was difficult to detect, suggesting that Per8p is a low abundance protein even in methanol-grown *H. polymorpha* where peroxisomes are prominent (Fig. 1 *A*). To confirm the identity of the 31-kD species as Per8p, extracts prepared from methanol-grown cells of the $P_{MOX}$-*PER8* strain were examined and showed a very prominent band at 31 kD (Fig. 11 *A*, lane *3*). Subsequent affinity purification of the serum resulted in a preparation that reacted specifically with Per8p (Fig. 11 *A*, lanes *5* and *6*).

To determine the subcellular location of Per8p, immunocytochemical experiments were performed on sections from methanol-grown cells of wild-type and $P_{MOX}$-*PER8* strains. In both, specific labeling was found almost exclusively on or near the peroxisomal membranes (Fig. 10, *B* and *D*). Moreover, the gold particles appeared to be concentrated on the membranes of small (presumably newly formed) organelles, a feature that was particularly evident in $P_{MOX}$-*PER8* cells (Fig. 10 *D*).

The location of Per8p on or near peroxisomal membranes was characterized biochemically. Peroxisomes from methanol-grown wild-type cells that had been purified by sucrose density gradient centrifugation were examined by immunoblotting and were found to contain Per8p. Samples of these purified peroxisomes were extracted with either triethanolamine or sodium carbonate. Following triethanolamine treatment and centrifugation at 30,000 *g*, AOX and CAT were located mostly in the supernatant, whereas Per8p was found in the membrane pellet (Fig. 11 *B*, lanes *1* and *2*), suggesting that Per8p was associated with the peroxisomal membrane. After carbonate extraction and centrifugation at 100,000 *g*, Per8p remained in the membrane pellet (Fig. 11 *B*, lanes *3* and *4*), suggesting that Per8p was an integral peroxisomal

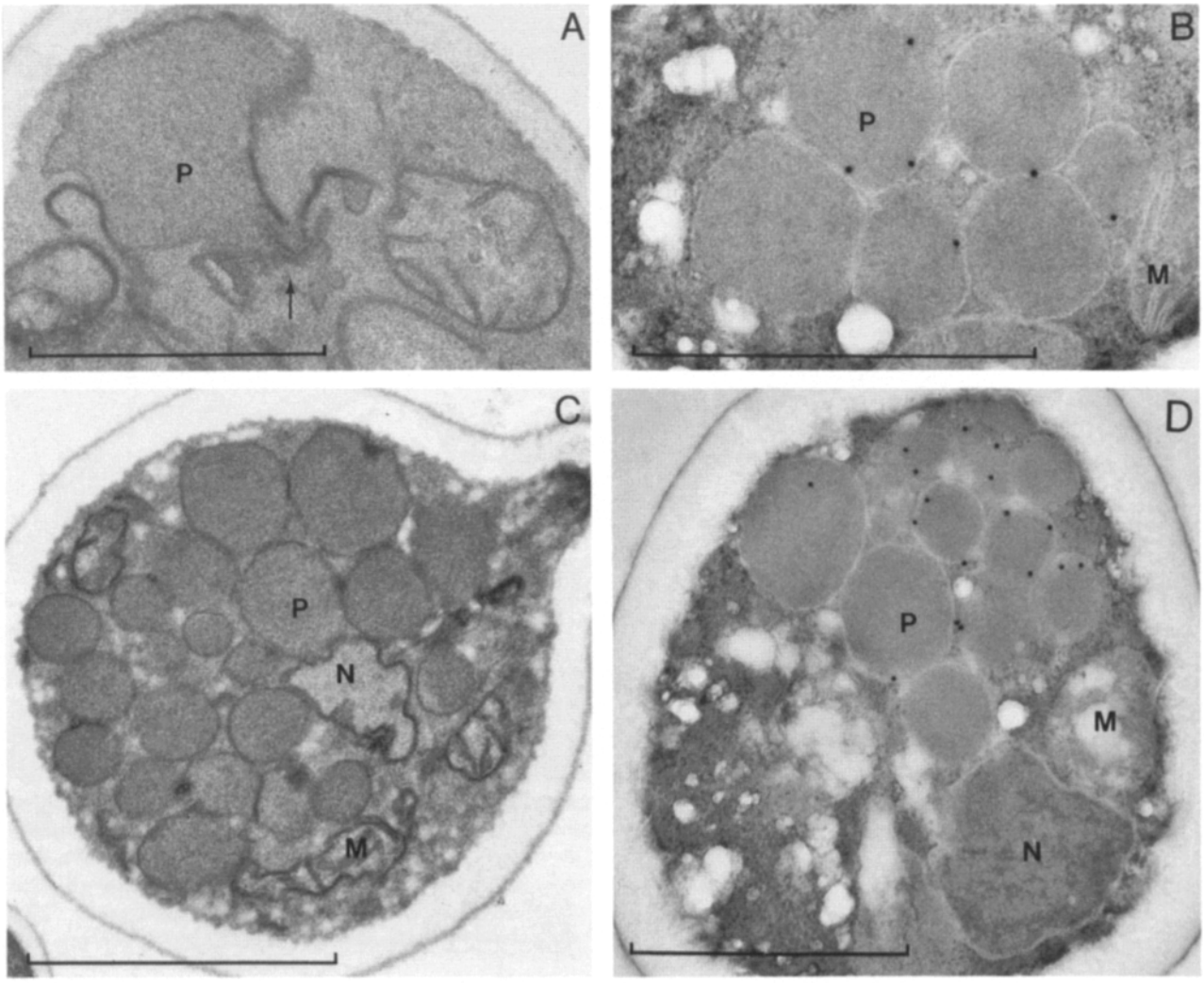



*Figure 10.* (*A*) Electron micrograph showing possible peroxisome fission event in the $P_{MOX}$-*PER8* strain 4 h after shift to methanol medium. Subcellular localization of Per8p in methanol-grown wild-type (*B*) and $P_{MOX}$-*PER8* strain (*D*) after immuno-gold labeling using affinity-purified antibodies against Per8p. (*C*) $P_{MOX}$-*PER8* cell after 24 h in methanol medium showing abnormally large number of peroxisomes. Bar, 1.0 μm.

membrane protein. To further support this conclusion, samples of pellet fractions resulting from differential centrifugation of methanol-grown $P_{MOX}$-*PER8* cells were subjected to extraction with urea or Triton X-114. After Triton X-114 extraction and centrifugation at 300 *g*, Per8p partitioned from the aqueous phase to the detergent phase as expected for a membrane protein (Fig. 11 *B*, lanes *7* and *8*). After urea extraction and centrifugation at 250,000 *g*, Per8p was mostly in the membrane pellet (Fig. 11 *B*, lanes *5* and *6*).

## *Discussion*

Previously, we described the isolation and preliminary characterization of the chemically induced *per8-1* strain as one of a large number of *H. polymorpha* mutants affected in peroxisome biogenesis (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In this report, we describe the further characterization of the *per8-1* strain and its comparison to an in vitro-constructed *per8*Δ null mutant. Both *per8-1* and *per8*Δ alleles result in *H. polymorpha* strains without any peroxisome-like structures, a phenotype we have named Per⁻. Their apparent total absence contrasts with some mammalian peroxisome-deficient mutants where small abnormal vesicular structures, believed to be peroxisomal remnants, are observed (Santos et al., 1988). Consistent with the lack of peroxisomes in *per8* mutants, all peroxisomal matrix enzymes that we examined (AOX, CAT, DHAS, and AMO) are located in the cytosol. To date, two peroxisomal targeting signals, PTS1 and PTS2, have been defined (de Hoop and AB, 1992; Subramani, 1993). The three methanol-pathway enzymes, AOX, CAT, and DHAS, are believed to be imported via a PTS1-type system since each ends in a tripeptide with similarity to the prototypical PTS1, SKL (Didion and Roggenkamp, 1992; Hansen et al., 1992). Specifically, the carboxy-terminal amino acids are ARF for AOX, SKI for CAT, and NKL for DHAS. For each of these enzymes, it has been shown that deletion of the three terminal residues results in a mutant enzyme that is cytosolic. In

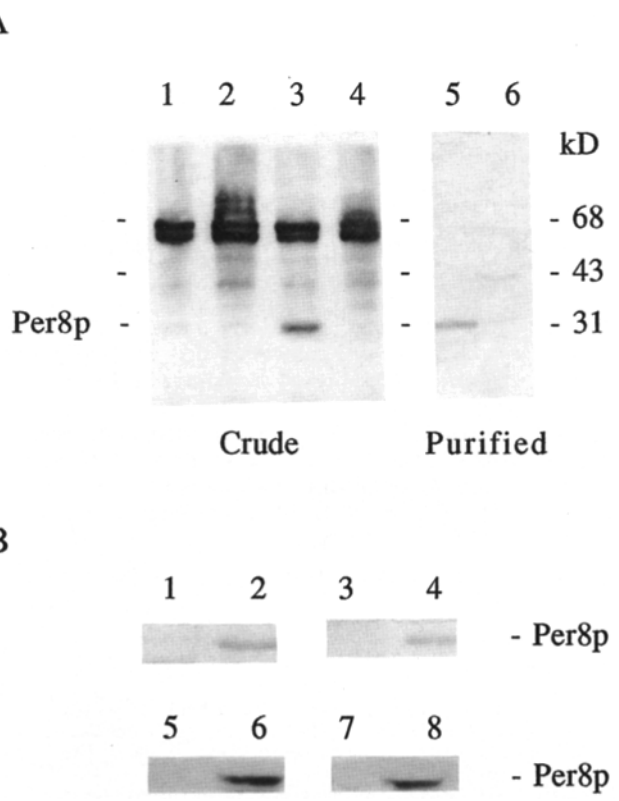



*Figure 11.* (*A*) Immunoblots showing that both crude and affinity-purified anti-Per8p antisera react with a 31-kD protein species in extracts prepared from methanol-induced cells. Lanes *1–4* each contain 10 μg of protein extract, and lanes *5* and *6* each contain 30 μg of protein extract from the following *H. polymorpha* strains: (lane *1*) wild type; (lane *2*) *per8-1* strain; (lanes *3* and *5*) $P_{MOX}$-*PER8* strain; (lanes *4* and *6*) *per8*Δ strain. (*B*) Distribution of Per8p after extraction of peroxisomes. In lanes *1–4*, 30 μg of purified peroxisomes isolated from methanol-grown wild-type cells were extracted with 20 mM triethanolamine, pH 7.8, (lanes *1* and *2*) or 0.1 M sodium carbonate, pH 11, (lanes *3* and *4*). In lanes *5–8*, 28 μg of postdifferential centrifugation pellet fraction from methanol-grown $P_{MOX}$-*PER8* cells were extracted with 0.5% Triton X-114 (lanes *5* and *6*) or 6 M urea (lanes *7* and *8*). After extraction, samples were centrifuged, and supernatant (odd numbered lanes) and pellet (even numbered lanes) fractions (for Triton extractions, aqueous and detergent phase fractions) were immunoblotted with anti-Per8p antibodies.

addition, the carboxy termini of AOX and DHAS were demonstrated to be sufficient for peroxisomal targeting when appended to a non-peroxisomal protein (Hansen et al., 1992). Thus, it appears that *per8* mutants cannot import PTS1-type enzymes. AMO is required for utilization of methylamine as a nitrogen source and has recently been shown to be imported via an amino terminal PTS2 sequence (Faber et al., 1995). Thus, the failure of *per8* mutants to import AMO suggests that PTS2-containing proteins are also not imported. Furthermore, the fact that methylamine-grown *per8* cells are also peroxisome-deficient indicates that this defect is not limited to the organelles of cells in methanol, but is a general one affecting peroxisome biogenesis under all conditions. Overall, our studies of *per8* mutants demonstrate that they are profoundly and specifically affected in peroxisome biogenesis and therefore, that the *PER8* gene is essential for this process.

To gain insight into the structure of the PER8 gene product, the *PER8* locus was cloned. The sequence of Per8p predicts a novel polypeptide of 295 amino acids (~34 kD) with no overall sequence similarity to any other protein in the data bases. (However, the *S. cerevisiae* peroxisome biogenesis protein Pas4p shows strong similarity to Per8p over its entire length [38% identity and 50% similarity] suggesting it may be the functional homologue of Per8p [Kunau, W.-H., personal communication].) The carboxy-terminal region of Per8p contains a segment of 39 residues that is a perfect match to the C3HC4 motif, a subgroup within the cysteine-rich zinc-finger domain family (Freemont et al., 1991; Coleman, 1992). For most of the approximately 30 proteins in this group, the C3HC4 region is thought to be involved in nucleic acid binding. However, in no case has the binding of zinc by a C3HC4 motif actually been demonstrated, and DNA binding by the motif is only surmised from the protein's function, such as DNA repair (Rad18p; Jones et al., 1988), transcriptional regulation (PML; Kakizuka et al., 1991), or DNA binding (MEL-18; Tagawa et al., 1990). Per8p belongs to a subset of proteins within the C3HC4 subgroup whose members are not located in the nucleus and therefore, probably do not interact with DNA. These proteins include: the *S. cerevisiae* vacuolar membrane-associated proteins Vps11p/Pep5p/End1p (Dulic and Riezman, 1989) and Vps18p/Pep3p (Robinson et al., 1991); the mammalian peroxisomal integral membrane protein PAF1p (Shimozawa et al., 1992); and the mouse peripheral membrane postsynaptic 43-kD protein (Froehner, 1989). These proteins are further distinguished from other C3HC4 subgroup members by the carboxy-terminal location of the motif. It has been suggested that the C3HC4 motif in these nonnuclear proteins is involved in protein–protein interactions (Freemont et al., 1991; Kunau et al., 1993). With regard to Per8p, this hypothesis is consistent with recent genetic evidence that the PER8 gene product interacts with at least three other PER products (Titorenko et al., 1993).

Per8p behaves in biochemical experiments as a peroxisomal integral membrane protein. After discontinuous sucrose centrifugation of crude organelle preparations, Per8p is present in peroxisomal fractions. Furthermore, after extraction with sodium carbonate, urea or Triton X-114, Per8p remained associated with the membrane fraction, indicating that Per8p is an integral membrane protein. These results were further supported by immunocytochemical experiments which showed that Per8p is exclusively located on the peroxisome membrane.

The failure of *per8* mutants to import peroxisomal enzymes might be taken as evidence that Per8p is a component of the peroxisome protein import machinery. However, defects in other peroxisome functions such as the duplication, proliferation or segregation should also result in the absence of peroxisomes and, as a consequence, a cytosolic location for peroxisomal enzymes. An important clue to the possible function of Per8p comes from ultrastructural studies of an *H. polymorpha* strain that overexpresses *PER8* under control of the alcohol oxidase promoter $P_{MOX}$. Upon shift of this strain from glucose to methanol, a greatly enhanced proliferation of peroxisomes occurs. The extent of this proliferation is not seen in wild-type cells and thus, ap-

pears to be a response to the increased amount of Per8p. The proliferation events in the overexpression strain take place in bursts at approximately 5 and 10 h after shift. Such synchronous bursts are not observed in wild-type cells and are not readily explained. Perhaps in wild-type cells, the concentration of Per8p is normally the limiting factor controlling the initiation of proliferation. When Per8p is overexpressed, another factor becomes limiting and thereby, determines the timing of proliferation events. One possibility for a factor that might synchronize proliferation is suggested by the observation that peroxisomes appear to need to reach a critical volume or mature size before budding begins (Veenhuis and Harder, 1991). Thus, a factor that senses peroxisome volume may be limiting. A second possibility is suggested by the time between proliferation cycles which is approximately the same as the generation rate of the strain in methanol medium. Thus, a factor that links proliferation to the cell cycle may be limiting initiation events in the Per8p overexpression strain. Most importantly, the hyperproliferative response of peroxisomes to high levels of Per8p indicates that the protein is a component of the machinery that controls peroxisome proliferation in this yeast.

A role for Per8p in proliferation is also supported by our observation that the protein is concentrated in the membranes of small and presumably newly formed peroxisomes. Peroxisome budding is similar in overall appearance to the formation of vesicles during endocytosis and secretion. During the latter vesicle formation events, components of the fission machinery are known to concentrate in or around the membranes of emerging vesicles (Pryer et al., 1992). Although the mechanisms driving peroxisome and secretory-vesicle budding may be very different at the molecular level, it is likely that both involve the presence of specific machinery components at the site of the emerging vesicle. Therefore, the presence of Per8p predominantly in newly formed peroxisomes relative to larger older organelles is consistent with the involvement of the protein in peroxisome proliferation.

Several considerations lead us to suspect that Per8p may be involved in at least one other aspect of peroxisome biogenesis. If Per8p was solely involved in the proliferative response to environmental changes but not in the peroxisome duplication or segregation processes responsible for maintaining the organelle through cell division cycles, *per8* cells would be predicted to continue to contain at least one small peroxisome. Upon methanol induction, one would expect the small organelle to increase in size through import of matrix proteins. This expectation comes from studies on the effect of expressing AOX and DHAS in glucose-grown *H. polymorpha.* When either of these enzymes are expressed at a high level in glucose medium, the one or two small peroxisomes that normally exist in these cells are observed to increase in volume but not number (Distel et al., 1988; Gödecke et al., 1989; Roggenkamp et al., 1989). The same effect, peroxisome growth without proliferation, would also be expected of a mutant that is specifically defective in proliferation. In contrast, *per8* cells appear to have no peroxisomes and, upon methanol induction, peroxisomal enzymes are located in the cytosol. This suggests that, in addition to environmentally induced proliferation, Per8p is also required for some other aspect of peroxisome biogenesis.

In conclusion, the hyperproliferative response of peroxisomes to *PER8* overexpression and the concentrated presence of Per8p in the membrane of newly formed organelles indicate that Per8p is part of the machinery controlling peroxisome proliferation in *H. polymorpha.* The similarity of Per8p to Pas4p, a protein required for peroxisome biogenesis and oleate growth in *S. cerevisiae* (W.-H. Kunau, personal communication), strongly suggests that these proteins are not pathway- or organism-specific products, but are a universal component of the organelle's proliferative machinery.

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